Validation of TB-FPA

Fernando Rivera[1], Michael Gilsdorf[2], Miladin Kostovic[3]

The accurate identification of bovines infected with *Mycobacterium bovis* (BTB) forms the foundation of an effective disease control and eradication program. Accurately identifying infected animals is challenging because currently available BTB tests are not 100% sensitive or specific and can lead to false-negative results for some infected animals, especially during the early and late stages of the disease. This study assessed the BTB fluorescence polarization assay's (BTB-FPA) ability to correctly identify BTB-non-infected animals and to detect BTB-anergic animals when compared to the caudal fold test (CFT), gamma-interferon (IFN-γ), and postmortem results.

Test-negative and test-positive bovine results were compared between BTB-FPA, CFT, CCT, and IFN-γ. The BTB-FPA displayed a 100% correlation for 453 test-negative animals. On 100 animals tested from a BTB-infected herd, CFT, CCT, IFN-γ, and BTB-FPA results varied significantly. There was disagreement between CFT and IFN-γ results for 56 animals, and all three test results only agreed on 10 animals. The BTB-FPA identified three lesioned animals out of 93 (mean of 3% positive) that were negative according to CFT and IFN-γ but positive according to postmortem results (i.e. visible BTB lesions and positive cultures). These results indicate that the BTB-FPA is suitable in identifying BTB-anergic animals in an infected herd.

Introduction

Tuberculosis (TB) is a 3 million-year-old zoonotic disease. Ancestral *Mycobacterium tuberculosis* mutated into multiple strains including *Mycobacterium bovis*, which causes bovine tuberculosis (BTB). BTB can be transmitted between animals and humans, and BTB control/eradication programs have been in place since the late nineteenth/early twentieth century. A purified protein derivative (PPD) of tuberculin has been developed and used to test animals and humans for many years. Because of its specificity, the caudal fold test (CFT) is the primary PPD test used to diagnose BTB in bovines. The CFT measures cell-mediated delayed-type hypersensitivity against bovine PPD when injected into an animal. The CFT is not ideal because it is lengthy (time-to-answer is 72 hours), and its sensitivity is dependent on several factors that often cause it to be less sensitive. These factors can be related to the immunological response (early infection, anergy, or concurrent immunosuppression), the PPDs being used (expired product, product stored under inappropriate conditions, manufacturing errors, low potency, etc.) or methodology (doses, site of injection, inexperience, etc.) and can cause false-negative results. In contrast, co-infection or pre-exposure to other related non-tuberculous mycobacteria have been reported as potential causes of false-positive results due to the similar antigenic composition of these bacteria.

Not all PPD tuberculin test antigens are equivalent. Consistent biological potency among tuberculin production cycles is critical for the outcome of this intradermal test. Significant differences in the number of animals testing positive have been reported using high- versus low-potency tuberculin. For this reason, each government regulates the production of their PPD tuberculin. In addition, countries who are members of the World Health Organization (WHO), the International Office of Epidemiology (OIE), and/or the European Economic Community (EU) have established worldwide legal standards for tuberculin production, potency assay performance, and intradermal tests for bovines. Importing and exporting countries are required to use these tests when trading or moving livestock locally or internationally. Alternative BTB tests such as the single intradermal comparative cervical PPD test (SICCT or CCT) and the interferon-γ test (IFN-γ) have been developed and used in combination with the CFT as

1. Zoolab Laguna, Torreon, Coahuilla, Mexico
2. International Animal Health Solutions, Grad, Maryland, U.S.A.
3. Ellie LLC, Germantown, Wisconsin, U.S.A.

Corresponding author: miladin@ellielab.com

supplemental or confirmatory tests. Whereas the CFT measures an animal's hypersensitivity response to mammalian PPD, the CCT compares both mammalian (bovine) and avian PPD tuberculin responses. This increases specificity because avian tuberculosis (ATB) can cause CFT cross-reactions, and ATB does not cause the severe disease that M. bovis causes in cattle.

IFN-γ is used to measure an animal's cellular response to M. bovis infection. Measuring IFN-γ has some advantages over the CFT and CCT. For example, animals only need to be handled once, the test can be repeated immediately, and its interpretation is less subjective. However, IFN-γ also has limitations that include lower specificity, especially in younger animals; the need for laboratory processing; substantial laboratory expertise; and significant test costs.

Not all BTB-infected animals develop a detectable hypersensitive or cellular response. These animals are referred to as anergic animals because they are not detected with standard BTB tests. Antibody development occurs most often in animals with a more advanced disease state, and these animals are more likely to be infectious and transmit the disease to herd mates. It has been theorized that these animals fail to develop a hypersensitivity response because their body is overwhelmed with circulating tuberculin antigens. Therefore, the CFT and CCT fail to detect anergic animals.

No BTB test or combination of tests can provide 100% sensitivity (the proportion of infected animals detected by the test) and 100% specificity (the proportion of non-infected animals that are diagnosed as negative). The estimated sensitivity and specificity between various BTB tests can range from 55% to 99%.

Many efforts have been made to develop a highly sensitive and specific BTB antibody test similar to those used in other bacterial disease eradication and control programs, such as brucellosis. However, the sensitivity of such tests depends on the infected animals developing an antibody response. This seems to occur only in the latter stages of the disease.

For the past 20 years, Ellie has been investigating how and when BTB-infected bovine develop antibody responses. As a recent milestone in this investigation, Ellie developed a BTB-FPA, and this paper reports its preliminary performance in testing bovines.

Materials and Methods

Herds

To detect whether the TB FPA can detect anergic animals, individual blood samples were taken from bovines from two different herds. In one herd, which was located in Serbia, samples were collected from 453 cattle that were first deemed to be test-negative by CFT and re-tested using the BTB-FPA.

A second herd, known to be BTB-affected, was located in Mexico. One hundred CFT-positive bovines were tested using the BTB-FPA. After testing the animals, they were euthanized, and necropsies were conducted. All animals exhibited lesions consistent with a BTB infection.

Sample testing was conducted using a single-blind study design in which sample information (e.g. herd number, ear tag, and other laboratory test results) was withheld from technical staff.

The BTB-FPA test result for each animal was compared against the CFT, IFN-γ, and postmortem results from the same animal. Infection was defined as a positive CFT result (standard interpretation), a positive IFN-γ result, a visible lesion at slaughter, and/or bacteriological confirmation result (positive by histology and/or microbiological culture).

Results

All 453 CFT-negative bovines tested negative for BTB using the BTB-FPA, (100% specificity). A comparison of BTB-FPA, CFT, IFN-γ, and postmortem results generated from the 100 animals in the infected group are presented in Table 1. Of the 100 animals diagnosed as positive via postmortem results, 28 generated false-negative results according to CFT, and another 28 also generated false-negative results according to IFN-γ measurements. The BTB-FPA showed false-negative results for 85 of the 100 bovines. However, the BTB-FPA also detected three lesioned, BTB-infected anergic animals that tested negative according to CFT and IFN-γ. In addition, BTB-FPA, CFT, and IFN-γ results agreed on 10 animals. The BTB-FPA identified two other positive animals that were deemed positive by only one of the other tests.

Table 1. Results of testing 100 dairy cows in Laguna, Mexico. Age is presented in months. Results of the INF-γ test (Bovigam) are labeled as "TB" when positive for BTB and "PTB" when positive for avian TB.

COW	AGE	CFT	GAMMA	BTB-FPA
1	37.1	+	–	–
2	57.7	+	TB	–
3	27.5	+	PTB	–
4	47.1	–	–	+
5	45.9	+	TB	–
6	71.4	+	–	–
7	44.2	–	TB	–
8	56.2	+	–	
9	60	+	TB	–
10	66.1	+	PTB	+
11	74	+	PTB	–
12	50.3	–	TB	–
13	52.1	–	–	–
14	51.7	–	PTB	–
15	67.6	+	TB	–
16	50.9	+	TB	
17	61.3	+	PTB	–
18	59.2	–	TB	–
19	59.7	+	PTB	+
20	85.4	+	TB	
21	75.6	+	TB	–
22	84	+	PTB	–
23	56.8	+	TB	–
24	79.6	+	TB	–
25	92	–	–	–
26	59.6	+	TB	–
27	51.3	+	TB	–
28	89.7	+	TB	–
29	84.2	+	–	–
30	56.3	–	PTB	–
31	69.9	+	PTB	–
32	59.2	+	TB	–
33	67.7	+	PTB	–
34	37.7	–	TB	+
35	100.5	+	TB	–
36	75.9	–	PTB	–
37	46.7	+	TB	–
38	59.1	–	–	+
39	52.6	+	PTB	+
40	59.1	+	TB	–
41	60.6	+	–	–
42	74.9	+	TB	–
43	83.7	+	TB	–
44	75.1	–	TB	–
45	84.5	+	TB	+
46	26.3	+	–	–
47	57.9	–	TB	–
48	64.3	–	PTB	–
49	67.9	–	–	
50	86.3	+	TB	–

COW	AGE	CFT	GAMMA	BTB-FPA
51	102.3	–	–	–
52	66.8	+	TB	–
53	65.8	+	–	
54	111.3	+	TB	–
55	48.3	–	–	–
56	66.3	+	–	–
57	86.1	+	TB	+
58	34.9	+	TB	+
59	85.9	–	PTB	–
60	46.5	+	TB	+
61	87.4	+	PTB	–
62	89.7	+	TB	
63	82.7	+	TB	–
64	85.3	+	TB	–
65	48.8	+	PTB	+
66	74.4	+	–	–
67	49.7	+	–	–
68	61.5	+	TB	–
69	46.8	–	–	–
70	56	–	TB	–
71	82.4	+	–	+
72	81.7	+	–	–
73	55.8	+	TB	–
74	82.2	+	TB	–
75	44.9	–	–	–
76	48.5	–	–	–
77	48.5	+	–	–
78	84.5	+	TB	–
79	84	–	–	
80	47	+	TB	+
81	72.8	+	TB	–
82	95.8	–	–	–
83	86	+	TB	–
84	96.8	+	TB	+
85	74.6	+	TB	–
86	58.1	+	TB	–
87	72.5	+	TB	–
88	79.9	+	TB	–
89	45.3	+	TB	–
90	37.5	+	TB	–
91	37.4	+	TB	–
92	32	+	PTB	–
93	38.6	–	TB	–
94	71.6	+	PTB	–
95	49.9	+	–	–
96	29.8	–	–	–
97	43.9	–	TB	–
98	43.5	+	TB	–
99	35.4	+	–	–
100	34.7	–	–	+

Discussion

The existence of BTB anergic animals makes it difficult to diagnose and control *M. bovis*. Due to the particular and complex characteristics of BTB, there is a growing perception that no single testing method, per se, is sufficient for detecting all reactive animals at every stage of infection. Therefore, a manifold approach using several currently available methods should be applied. Modern approaches to diagnosis and control BTB should include bacteriological, molecular, histopathological, and immunological assays due to the indications, advantages, and disadvantages of each method.

In this study, the number of postmortem-positive animals that were also positive according to the BTB-FPA was very low. Obviously, this test should not be used by itself to diagnose BTB infection in a herd. However, if it is used as part of a multidisciplinary approach in conjunction with CFT and IFN-γ, these data suggest that additional BTB-infected animals can be detected by the BTB-FPA that would otherwise go undetected. Using these tests in parallel would eliminate the disease from the herd more quickly.

There is no data available at this time that shows the percentage of anergic animals the BTB-FPA detects. However, each anergic animal that is removed from the herd prevents that animal from transmitting BTB to its herd mates.

Conclusion

Diagnosing BTB in bovines is not a straightforward process. Many governments use a combination of tests to ensure that the best viable options are used to identify the disease and remove infected cattle from the farm. However, interpreting the results of each testing regime can vary depending on the disease situation in the region and on the farm. The preliminary results of this study indicate that the BTB-FPA can be used to detect anergic animals that the other BTB diagnostic tests cannot. Additional studies are needed to gather more data and confirm the results of these studies.

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